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# PROVISIONAL APPLICATION FOR PATENT COVER SHEET

**This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).**

| INVENTOR(S) | | |
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☐ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

**TITLE OF THE INVENTION (280 characters max)**

METHOD FOR THE DETECTION OF CYTOSINE METHYLATION IN A DNA SAMPLE

**CORRESPONDENCE ADDRESS**

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**ENCLOSED APPLICATION PARTS *(check all that apply)***

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☐ Application Data Sheet. See 37 CFR 1.76

**METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)***

☐ Applicant claims small entity status. See 37 CFR 1.27.

☒ A check or money order is enclosed to cover the filing fees

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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*Respectfully submitted,*

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## USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

# Method for the detection of cytosine methylation in a DNA sample

## FIELD OF THE INVENTION

The present invention relates generally to a method of determining the DNA methylation status of CpG sites in a given locus. In particular, this invention relates to the use of DNA amplification techniques including a transcription step for the detection of cytosin methylation patterns, especially to the use of DNA-NASBA (Nucleic Acid Sequence Based Amplification) for sensitive detection of DNA-methylation. In a preferred embodiment the invention relates to improvement of sensitivity of detection of DNA-methylation by combining bisulfite conversion of DNA, which transfers differential Cytosine-methylation into the base difference Uracil - Cytosine, and amplification by NASBA, which is a cyclic process includes a transcription step as well as use of RNase H, which specifically digests the RNA-strand in a RNA-DNA-heteroduplex. The latter property can be used for improvement of sensitivity of detection of DNA-methylation by including a DNA-oligonucleotide reverse complementary to a region within the amplicon representing methylation state of background DNA. Thereby amplification of background DNA is impaired, while amplification of the methylation state representing DNA to be investigated remains unchanged. Only these molecules will contribute to the final detection signal.

## BACKGROUND OF THE INVENTION

In mammals DNA is methylated only at cytosines located 5' to guanosine in the CpG dinucleotide. This modification has important regulatory effects on gene expression, especially when involving CpG rich areas, known as CpG islands, located in

the promoter regions of many genes. While almost all gene-associated islands are protected from methylation on autosomal chromosomes, extensive methylation of CpG islands has been associated with transcriptional inactivation of selected imprinted genes, genes related to cell differentiation and genes on the inactive X-chromosome of females. Abberant methylation of normally unmethylated CpG islands has been described as a frequent event in immortalized and transformed cells, and has been associated with transcriptional inactivation of defined tumor suppressor genes in human cancers.

Human cancer cells typically contain somatically altered genomes, characterized by mutation, amplification, or deletion of critical genes. In addition, the DNA template from human cancer cells often displays somatic changes in DNA methylation (E. R. Fearon, et al., Cell, 61:759, 1990; P. A. Jones, et al., Cancer Res., 46:461, 1986; R. Holliday, Science, 238:163, 1987; A. De Bustros, et al., Proc. Natl. Acad. Sci., USA, 85:5693, 1988; P. A. Jones, et al., Adv. Cancer Res., 54:1, 1990; S. B. Baylin, et al., Cancer Cells, 3:383, 1991; M. Makos, et al., Proc. Natl. Acad. Sci., USA, 89:1929, 1992; N. Ohtani-Fujita, et al., Oncogene, 8:1063, 1993). However, the precise role of abnormal DNA methylation in human tumorigenesis has not been established.

A CpG rich region, or "CpG island", has been identified at 17p13.3, which is aberrantly hypermethylated in multiple common types of human cancers (Makos, M., et al., Proc. Natl. Acad. Sci. USA, 89:1929, 1992; Makos, M., et al., Cancer Res., 53:2715, 1993; Makos, M., et al., Cancer Res. 53:2719, 1993). This hypermethylation coincides with timing and frequency of 17p losses and p53 mutations in brain, colon, and renal cancers. Silenced gene transcription associated with hypermethylation of the normally unmethylated CpG islands has been implicated as an alternative mechanism to mutations of

coding regions for inactivation of tumor suppressor genes (Baylin, S. B., et al., Cancer Cells, 3:383, 1991; Jones, P. A. and Buckley, J. D., Adv. Cancer Res., 54:1-23, 1990). This change has now been associated with the loss of expression of VHL, a renal cancer tumor suppressor gene on 3p (J. G. Herman, et al., Proc. Natl. Acad. Sci. USA, 91:9700-9704, 1994), the estrogen receptor gene on 6q (Ottaviano, Y. L., et al., Cancer Res., 54:2552, 1994) and the H19 gene on 11p (Steenman, M. J. C., et al., Nature Genetics, 7:433, 1994).

De novo methylation of the Rb gene has been demonstrated in a small fraction of retinoblastomas (Sakai, et al., Am. J. Hum. Genet., 48:880, 1991 Expression of a tumor suppressor gene can be abolished by de novo DNA methylation of a normally unmethylated 5' CpG island (Issa, et al., Nature Genet., 7:536, 1994; Herman, et al., supra; Merlo, et al., Nature Med., 1:686, 1995; Herman, et al., Cancer Res., 56:722, 1996; Graff, et al., Cancer Res., 55:5195, 1995; Herman, et al., Cancer Res., 55:4525, 1995).

Different methods developed to date for detection of methylated cytosine depend upon cleavage of the phosphodiester bond alongside cytosine residues, using either methylation-sensitive restriction enzymes or reactive chemicals such as hydrazine which differentiate between cytosine and its 5-methyl derivative. The use of methylation-sensitive enzymes suffers from the disadvantage that it is not of general applicability, since only a limited proportion of potentially methylated sites in the genome can be analyzed. Genomic sequencing protocols which identify a 5-MeC residue in genomic DNA as a site that is not cleaved by any of the Maxam Gilbert sequencing reactions, are a substantial improvement on the original genomic sequencing method, but still suffer disadvantages such as the requirement for large amount of genomic DNA and the difficulty in detecting a gap in a sequencing ladder which may contain

bands of varying intensity.

Mapping of methylated regions in DNA has relied primarily on Southern hybridization approaches, based on the inability of methylation-sensitive restriction enzymes to cleave sequences which contain one or more methylated CpG sites. This method provides an assessment of the overall methylation status of CpG islands, including some quantitative analysis, but is relatively insensitive, requires large amounts of high molecular weight DNA and can only provide information about those CpG sites found within sequences recognized by methylation-sensitive restriction enzymes. A more sensitive method of detecting methylation patterns combines the use of methylation-sensitive enzymes and the polymerase chain reaction (PCR). After digestion of DNA with the enzyme, PCR will amplify from primers flanking the restriction site only if DNA cleavage was prevented by methylation. Like Southern-based approaches, this method can only monitor CpG methylation in methylation-sensitive restriction sites. Moreover, the restriction of unmethylated DNA must be complete, since any uncleaved DNA will be amplified by PCR yielding a false positive result for methylation. This approach has been useful in studying samples where a high percentage of alleles of interest are methylated, such as the study of imprinted genes and X-chromosome inactivated genes. However, difficulties in distinguishing between incomplete restriction and low numbers of methylated alleles make this approach unreliable for detection of tumor suppressor gene hypermethylation in small samples where methylated alleles represent a small fraction of the population.

Another method that avoids the use of restriction endonucleases utilizes bisulfite treatment of DNA to convert all unmethylated cytosines to uracil. The altered DNA is amplified and sequenced to show the methylation status of all CpG sites. However, this method is technically difficult, labor intensive and without

cloning amplified products, it is less sensitive than Southern analysis, requiring approximately 10% of the alleles to be methylated for detection.

To date, barring few exceptions (e.g., Zeschnigk M, Lich C, Buiting K, Doerfler W, Horsthemke B. (1997) A single-tube PCR test for the diagnosis of Angelman and Prader-Willi syndrome based on allelic methylation differences at the SNRPN locus. Eur J Hum Genet. 5: 94-8) the bisulfite technique is only used in research. Always, however, short, specific fragments of a known gene are amplified subsequent to a bisulfite treatment and either completely sequenced (Olek A, Walter J. (1997) The pre-implantation ontogeny of the H19 methylation imprint. Nat Genet. 3: 275-6) or individual cytosine positions are detected by a primer extension reaction (Gonzalgo ML and Jones PA. (1997) Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). Nucleic Acids Res. 25 :2529-31, WO 95/00669) or by enzymatic digestion (Xiong Z, Laird PW. (1997) COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res. 25: 2535-4).

Another technique to detect hypermethylation is the so-called methylation specific PCR (MSP) (Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB. (1996), Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A. 93: 9821-6). The technique is based on the use of primers that differentiate between a methylated and a non-methylated sequence if applied after bisulfite treatment of said DNA sequence. The primer either contains a guanine at the position corresponding to the cytosine in which case it will after bisulfite treatment only bind if the position was methylated. Or the primer contains an adenine at the corresponding cytosine position and therefore only binds to said DNA sequence after bisulfite treatment if the cytosine was

unmethylated and has hence been altered by the bisulfite treatment so that it hybridizes to adenine. With the use of these primers, amplicons can be produced specifically depending on the methylation status of a certain cytosine and will as such indicate its methylation state.

Another new technique is the detection of methylation via RealTime PCR, also known as MethyLightTM (WO 00/70090). With this technique it became feasible to determine the methylation state of single or of several positions directly during PCR, without having to analyze the PCR products in an additional step. It may also be referred to as Methylation Specific Detection Method of RealTime PCR amplification. The MethyLight™ assay is a high-throughput quantitative methylation assay that utilizes fluorescence-based real-time PCR technology (for example by using TaqMan.™ probes) that requires no further manipulations after the PCR step (Eads et al., Cancer Res. 59:2302-2306, 1999). Briefly, the MethyLight™ process begins with a mixed sample of genomic DNA that is converted, in a sodium bisulfite reaction, to a mixed pool of methylation-dependent sequence differences according to standard procedures (the bisulfite process converts unmethylated cytosine residues to uracil). Fluorescence-based PCR is then performed either in an "unbiased" (with primers that do not overlap known CpG methylation sites) PCR reaction, or in a "biased" (with PCR primers that overlap known CpG dinucleotides) reaction. Sequence discrimination can occur either at the level of the amplification process or at the level of the fluorescence detection process, or both.

Identification of the earliest genetic changes in tumorigenesis is a major focus in molecular cancer research. Diagnostic approaches based on identification of these changes are likely to allow implementation of early detection strategies and novel therapeutic approaches targeting these early changes might lead to more effective cancer treatment.

A method allowing sensitive detection, but so far not used in cancer diagnostics, is Nucleic Acid Sequence-Based Amplification (NASBA) NASBA, closely related to Self-Susatained Sequence Replication (3SR) or Transcription Mediated Amplification (TMA) is a technology for isothermal, transcription-based amplification of nucleic acids, originally designed for sensitive detection of RNA-sequences. In the meantime there are also single reports about successfully using DNA a template.

NASBA involves 3 enzymes: Reverse Transcriptase (RT), T7-DNA dependent RNA-polymerase and RNase H. A DNA-primer comprising the T7-promoter sequence at its 5'-end and a target specific sequence at the 3'-end anneals to the RNA-template (if using DNA, a denaturation step is needed in order to make the template strand accessible for the primer). The primer is extended by RT, thereby producing a (-)-cDNA-copy of the targeted RNA-sequence. The resulting RNA-DNA-heteroduplex is recognized by RNase H, which will specifically digest the RNA-strand (if DNA is used as a template another denaturation step is needed to separate strands). A second primer, reverse complementary to the 3'- end of the targeted sequence will bind to the single stranded (-)-cDNA, resulting in a dsDNA-molecule (the newly generated DNA-strand now comprising the sequence of the original RNA-strand) after extension by RT. At the double-stranded T7-promoter sequence T7-polymerase initiates multiple transcription of (-)-RNA-copies of this amplicon. Here NASBA enters the cyclic phase: The 2$^{nd}$ primer will bind to the (-)-RNA and subsequently be extended, which results in a RNA-DNA-heteroduplex.

NASBA is mainly used for sensitive detection of pathogens in bodyfluids. Here, RNA-sequences of pathogens (e.g. rRNA of bacteria or RNA-virus-sequences) are specifically amplified by

NASBA and resulting amplificates specifically detected by using different methods. These methods include use of fluorescently labeled Molecular Beacon-probes, which allow real time quantitation of amplification, or Hybridization Protection Assay (HPA) for specific, chemilumnescent end-point-detection of amplification products.

For determination of initial amounts of the target sequence a known amount of a calibrator fragment is included into each reaction, which has the same primer-binding sites like the target-RNA, but another sequence for specific detection. Calibrator signals are then compared to specific signals.

In contrast to the usual application of NASBA, where target sequences are unique to the specific pathogen, but not part of the human genome, DNA-methylation analysis requires sensitive and specific detection of a variant (usually the methylated version of a sequence) of DNA in a large background of another variant of this DNA (usually the unmethylated version of the sequence). Unfortunately, this kind of need for sensitivity and specificty is not adressed by the conventional NASBA.

When ensuring specific detection by using a probe-sequence, which is reverse-complementary to the sequence representing the methylated state (containing many CpG-positions), this will only be able to detct methylated DNA, when it comprises at least 5 %. This will not result in sufficient sensitivity for detection of few copies of methylated DNA within a up to 4000-fold background of unmethylated DNA. Therefore, sensitivity has to be increased.

This could be achieved by using primers, which are reverse complementary to the sequence representing the methylated state (containing many CpG-positions). But using such primers oftenly result in amplification of contaminating unconverted DNA. Additionally, such primers oftenly show stable hairpins, homo-

or heterodimers, which results in amplification of artifacts and reduction of available primer amounts.

STATMENT OF THE PROBLEM

The present invention will offer a sensitive method which is suitable for the detection of cytosine methylations in DNA samples.

This problem is solved by the provision of the embodiments as defined in the claims.

Therefore, the present invention, will solve the problem that current methods are unable to solve, i.e., to amplify in a targeted manner a DNA to be investigated („target nucleic acid") that is found in a body fluid, such as serum, when other, sequence-homologous DNA segments of different origin („background nucleic acids) are also present. The DNA to be investigated as well as the otherwise present nucleic acids, are usually amplified equally, since the primers used are not able to distinguish between the nucleic acids to be investigated and background nucleic acids. One possibility for distinguishing these DNAs, however, is by their different methylation patterns. Therefore the term „background nucleic acids" as used throughout this application refers to nucleic acids, which differ from the „target nucleic acids" by their methylation pattern. Throughout this amplification „background nucleic acid" also refers to the DNA and/or RNA copies, i.e. the complementary strands that were derived specifically from these DNA sequences differeing in their methylation pattern.

Before the present methods are described, it is to be understood that this invention is not limited to the particular methods, compositions, and cell lines described herein, as such methods, compositions, and cell lines may, of course, vary. It is also to be understood that the terminology used herein is

for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which is only defined by the appended claims.

As used herein, including the appended claims, singular forms of words such as "a," "an," and "the" include their corresponding plural referents unless the context clearly dictates otherwise. Thus, e.g., reference to "a cancer" includes one or more different cancers, reference to "a cell" includes one or more of such cells, and reference to "a method" includes reference to equivalent steps and methods known to a person of ordinary skill in the art, and so forth.

Prior to setting forth the invention it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references discussed above are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of its prior invention.

As used herein, the phrases "target nucleic acid" and "target sequence" are used interchangeably. Both phrases refer to a nucleic acid sequence, the presence or absence of which is desired to be detected. Target nucleic acid can be single-stranded or double-stranded and, if it is double-stranded, it may be denatured to single-stranded form prior to its detection

using methods, as described herein, or other well known methods. Additionally, the target nucleic acid may be nucleic acid in any form most notably DNA or RNA.

As used herein, "sample" refers to a substance that is being assayed for the presence of one or more nucleic acids of interest. The nucleic acid or nucleic acids of interest may be present in a mixture of other nucleic acids. A sample, containing the nucleic acids of interest, may be obtained in numerous ways. It is envisioned that the following could represent samples: cell lysates, purified genomic DNA, body fluids such as from a human or animal, clinical samples, food samples, etc.

As used herein, the phrases "target nucleic acid" and "target sequence" are used interchangeably. Both phrases refer to a nucleic acid sequence, the presence or absence of which is desired to be detected. Target nucleic acid can be single-stranded or double-stranded and, if it is double-stranded, it may be denatured to single-stranded form prior to its detection using methods, as described herein, or other well known methods. Additionally, the target nucleic acid may be nucleic acid in any form most notably DNA or RNA.

As used herein, the term "background-DNA" or "background nucleic acid" is used for nucleic acid sequences, which do not represent the methylation state to be investigated. These can be DNA-sequences or RNA-copies thereof.

As used herein, (+)-strand or -copy and (-)-strand or -copy describe the sense of a nucleic acid sequence compared to the target seqeunce. (+)-strands have the same sequence, while (-)-strands are reverse complement to the target-sequence.

As used herein, CpG-position refers to the base sequence CG, a base context, in whichCytosine is known to be differentially

methylated. After bisulfite conversion and amplification a former CpG-position can consist of the bases CG or TG or CA, depending on the methylation state.

As used herein, "amplification" refers to the increase in the number of copies of a particular nucleic acid target of interest wherein said copies are also called "amplicons" or "amplification products".

As used herein, "amplification components" refers to the reaction materials such as enzymes, buffers, and nucleic acids necessary to perform an amplification reaction to form amplicons or amplification products of a target nucleic acid of interest.

As used herein, "oligonucleotide" refers to a molecule comprising two or more deoxyribonucleotides or ribonucleotides, preferably more than three. The length of an oligonucleotide will depend on how it is to be used. The oligonucleotide may be derived synthetically or by cloning. Oligonucleotides may also comprise protein nucleic acids (PNAs). As used herein, "probe" refers to a known sequence of a nucleic acid that is capable of selectively binding to a target nucleic acid. More specifically, "probe" refers to an oligonucleotide designed to be sufficiently complementary to a sequence of one strand of a nucleic acid that is to be probed such that the probe and nucleic acid strand will hybridize under selected stringency conditions. Specific types of oligonucleotide probes are used in various embodiments of the invention. For example, FRET-probes describe one type of probe-systems designed to bind to a target nucleic acid of interest Thereby a detectable fluorescent "signal" will be generated.

As used herein, the terms "primer molecule" and "primer" are used interchangeably. A primer is a nucleic acid molecule, which possesses a chemical group at the 3' terminus that will

allow extension of the nucleic acid chain such as catalyzed by a DNA polymerase or reverse transcriptase.

As used herein, the phrase "amplification primer" refers to an oligonucleotide primer used for amplification of a target nucleic acid sequence.

As used herein, the term "T7-tailed-primer" or "T7-tail" describes the 5'-region of a primer oligonucleotide, which has a sequences serving as a promoter for T7 DNA-dependent RNA-polymerase. This is only an example of suitable promoter sequences, e.g. T3- or SP6-promoter could be used in combination with respective RNA-polymerases.

The phrase "primer extension," as used herein refers to the DNA polymerase induced extension of a nucleic acid chain from a free three-prime (3') hydroxy group thereby creating a strand of nucleic acid complementary to an opposing strand.

As used herein, the term "amplicon" refers to the product of an amplification reaction. An amplicon may contain amplified nucleic acids if both primers utilized hybridize to a target sequence. An amplicon may not contain amplified nucleic acids if one of the primers used does not hybridize to a target sequence. Thus, this term is used generically herin and does not imply the presence of amplified nucleic acids.

As used herein, the term "blocker" describes an DNA-oligonucleotide, that is modified in a way, that it cannot be used for primer extension. This can be achieved by different modifications of the 3'-terminus, e.g. phosphorylation. Additionally, the blocker sequence is reverse complementary to a sequence representing methylation state of the background-DNA, but not of the target to be detected.

As used herein, "hybridization" and "binding" are used interchangeably and refer to the non-covalent binding or "base pairing" of complementary nucleic acid sequences to one another. Whether or not a particular probe remains base paired with a polynucleotide sequence depends on the degree of complementarity, the length of the probe, and the stringency of the binding conditions. The higher the stringency, the higher must be the degree of complementarity, and/or the longer the probe for binding or base pairing to remain stable.

As used herein, the phrase "DNA polymerase" refers to enzymes that are capable of incorporating nucleotides onto the 3' hydroxyl terminus of a nucleic acid in a 5' to 3' direction thereby synthesizing a nucleic acid sequence. Examples of DNA polymerases that can be used in accordance with the methods described herein include, E. coli DNA polymerase I, the large proteolytic fragment of E. coli DNA polymerase I, commonly known as "Klenow" polymerase, "Taq" polymerase, T7 polymerase, Bst DNA polymerase, T4 polymerase, T5 polymerase, reverse transcriptase, exo-BCA polymerase, etc.As used herein, the term "RNase H" refers to an enzyme, which specifically hydrolyzes the phosphodiester bonds of RNA which is hybridized to DNA. This enzyme does not digest single or double-stranded DNA.

As used herein, the term "RNA-polymerase" refers to enzymes, which catalyze the synthesis of RNA in the 5´→ 3´-direction in the presence of a DNA template containing a suitable promoter. Examples of RNA polymerases that can be used in accordance with the methods described herein include, T7 RNA-polymerase, T3 RNA-polymerase, SP6 RNA-polymerase.

As used herein, the terms "detected" and "detection" are used interchangeably and refer to the discernment of the presence or absence of a target nucleic acid or amplified nucleic acid products thereof.

As used herein, the term "detecting the presence or absence of DNA methylation" refers to the detection of DNA methylation in the promoter region of one or more genes of a genomic DNA sample. The detecting may be carried out using any suitable method, including, but not limited to, those disclosed herein.

As used herein, the term "methylation profile" refers to a presentation of methylation status of one or more cancer marker genes in a subject's genomic DNA. In some embodiments, the methylation profile is compared to a standard methylation profile comprising a methylation profile from a known type of sample (e.g., cancerous or non-cancerous samples or samples from different stages of cancer). In some embodiments, methylation profiles are generated using the methods of the present invention. The profile may be presented as a graphical representation (e.g., on paper or on a computer screen), a physical representation (e.g., a gel or array) or a digital representation stored in computer memory.

As used herein, the term "non-human animals" refers to all non-human animals. Such non-human animals include, but are not limited to, vertebrates such as rodents, non-human primates, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, aves, etc.

As used herein, the term "subject suspected of having cancer" refers to a subject that presents one or more symptoms indicative of a cancer (e.g., a noticeable lump or mass). A subject suspected of having cancer may also have on or more risk factors. A subject suspected of having cancer has generally not been tested for cancer. However, a "subject suspected of having cancer" encompasses an individual who has received an initial diagnosis (e.g., a CT scan showing a mass) but for whom the sub-type or stage of cancer is not known. The term further includes people who once had cancer (e.g., an individual in remission).

As used herein, the term "subject at risk for cancer" refers to a subject with one or more risk factors for developing a specific cancer. Risk factors include, but are not limited to, genetic predisposition, environmental expose, preexisting non cancer diseases, and lifestyle.

As used herein, the term "stage of cancer" refers to a numerical measurement of the level of advancement of a cancer. Criteria used to determine the stage of a cancer include, but are not limited to, the size of the tumor, whether the tumor has spread to other parts of the body and where the cancer has spread (e.g., within the same organ or region of the body or to another organ).

As used herein, the term "providing a prognosis" refers to providing information regarding the impact of the presence of cancer (e.g., as determined by the diagnostic methods of the present invention) on a subject's future health (e.g., expected morbidity or mortality).

As used herein, the term "subject diagnosed with a cancer" refers to a subject having cancerous cells. The cancer may be diagnosed using any suitable method, including but not limited to, the diagnostic methods of the present invention.

Accordingly, the problem of the invention is solved by a method for the detection of cytosine methylation in a DNA sample - e.g. isolated from a body fluid of a subject suspected of having cancer - comprising the steps:

- a genomic DNA sample, which comprises the DNA to be investigated as well as background DNA is chemically treated in such a way that all of the unmethylated cytosine bases are converted to uracil, while the 5-methylcytosine bases remain unchanged;

- the chemically treated DNA sample is amplified by using a nucleic acid amplification method including a transcription step, whereby the amplification is conducted in the presence of at least one methylation-specific blocker, which binds to the background nucleic acid but substantially does not bind to the target nucleic acid, and

- the amplified products are analyzed and the methylation status in the DNA to be investigated is concluded from the presence or absence of an amplified product and/or from the analysis of additional CpG positions.

The essential advantage of the method described by the present invention is a methylation specific enrichment of sequences representing the methylation state to be detected. Thereby, sensitivity of detection is increased. This is especially advantageous, when large amounts of background DNA are present, which would otherwise compete for the same amplifcation and detection compounds with target DNA.

In one embodiment of the present invention, the method of the invention is further characterized in that the nucleic acid amplification is a nucleic acid sequence based amplification. Especially preferred is the method a self-sustaining sequence replication (3SR), a nucleic acid sequence based amplification (NASBA), a transcription-mediated amplification (TMA) or transcription from an artificially introduced promoter sequence.

Therefore, preferred nucleic acid amplification procedures are methods which include a transcription step. This applies to transcription-based amplification systems (TAS) like nucleic acid sequence based amplification (NASBA), transcription mediated amplification (TMA) and self-sustained sequence

replication (3SR) as well as amplification by transcrption from an artificially introduced promoter.

DNA, which is used as a template for these methods, has to be prepared from samples. This is preferably done by applying standard methods like lysis or heat treatment combined with phenol/ chloroform extraction or purification by using silica based purification systems.
In order to transfer methylation differences at cytosines within extracted DNA to amplifyable and detectable base differences appropriate methods have to be used. This is preferably done by a chemical treatment with bisulfite, which will convert unmethylated cytosines to uracil, while methylated cytosines are unaffected. Thereby base composition of DNA is changed: All cytosines except the ones, which were formerly methylated and which are typically found in the sequence context CpG will be converted to uracil, which will be replaced by thymine during amplification.

Amplification is done with amplification systems including a transcription step. Preferably NASBA is used. Bisulfite converted DNA, a (-)-primer 5'-tailed with the promoter sequence of a DNA-dependent RNA-polymerase, preferably the T7-RNA-polymerase, a (+)-primer, probe(s) and blocker(s), all specific for the targeted sequence are incubated. Incubation conditions will allow binding of the T7-tailed (-) primer to the (+)-strand of the target sequence, this will preferably include a short heating step for denaturation of secondary structures of all nucleic acid components as well as subsequent incubation at medium temperature, preferably 41°C.

T7-tailed primer will bind to a sequence, which does not include any CpG-position, thereby ensuring non-methylation-specific amplification. It will be extended by reverse transcriptase, generating a (-)-DNA-copy. Depending on sequence context of the target, another denaturation step will be needed

in order to make binding sites on (-)-DNA-strand accessible for the (+)-primer. Annealing of this primer and the (+)-blocker will occur during incubation at medium temperature, preferably 41°C, which is also the temperature for all folloing steps. (+)-primer will bind to a sequence, which does not contain any CpG-position. Blocker will preferably bind to a sequence representing methylation state of background DNA. It will not be extended as the 3'-end is modified in a way, which prevents covalent binding of nucleotides. Extension of the (+)-primer by reverse transcriptase results in a dsDNA-amplicon. If a blocker has bound, this will interfere with amplification, resulting in preferred amplification of sequences, which represent the methylation state to be detected. At the ds-promoter region T7-RNA-polymerase initiates transcription of multiple copies of (-)-RNA-strands. (+)-primer anneals to (-)-RNA-copies, blocker will mainly anneal to copies representing methylation state of background-DNA, which results in a DNA-RNA-heteroduplex wihin the annealing region. RNA within this structure will degraded by RNase H, which means, that these (-)-RNA-copies are no longer available for subsequent reactions. If RNA is not degraded, because the blocker did not not bind, (+)-primer will be extended, resulting in a (+)-DNA-copy. The RNA-template is degraded by RNase H. T7-tailed primer anneals to the (+)-DNA, gets extended and results in a dsDNA-amplicon. This is the starting point of the cyclic phase, as now again (-)-RNA-copies are transcribed and degraded or furhter amplified depending on the methylation state they are representing.
Detection of amplification products ((-)-RNA-copies) can be done by using various methods. Preferably, this will be done in the NASBA-reaction mixture during or at the end point of amplification by using a fluorecent probe, which will bind to a sequence representing the methylation state. This will preferably be the Molecular Beacon probe system or the LightCycler probe system.

It is preferred that the amplification is conducted in the second step in the presence of at least one additional oligonucleotide, which is called „blocking oligonucleotide" or „blocker" throughout this application. The blocker is characterized by the fact that its sequence comprises at least one 5'-CG-3' dinucleotide or 5'-TG-3' dinucleotide or 5'-CA-3', dinucleotide or any combination of these, wherein all of these dinucleotides represent CpG positions in the sequence to be blocked (background nucleic acid), which however appear either as TG or CG depending on their methylation status after conversion in step a) or as CA dinucleotide (representing an unmethylated CpG) when analyzing the complementary strand or „counter strand").

Wherein the blocking oligonucleotides hybridize to background RNA, the 5'-CA-3' dinucleotide binds to UG dinucleotide instead of a TG dinucleotide, wherein the thymine is replaced by uracil.

It is the binding of the DNA blocker to the background RNA, which is an RNA copy of the background DNA, which allows the specific elimination of background nucleic acids by the use of an enzyme such as RNAse H. Therefore it is especially preferred that the blocker is a DNA oligonucleotide.

It is a preferred embodiment of the method, that the blocker preferably binds to the background nucleic acid and adversely affects its amplification.

It is particularly preferred that this binding site of the blocking oligonucleotide, overlaps with the binding sites of the primers on the background nucleic acid and the blocking oligonucleotide hinders the binding of at least one primer oligonucleotide to the background nucleic acids.

In addition, it is particularly preferred that at least two blockers are utilized, whereby their binding sites each overlap in turn with the binding site of one primer on the background nucleic acid, and the blockers hinder the binding of both primer oligonucleotides to the background nucleic acids.

It is also particularly preferred that one of the additional oligonucleotides prevents the binding of the forward primer, while the other prevents the binding of the reverse primer.

It is particularly preferred that the additional oligonucleotides are present in at least five times the concentration of the primer oligonucleotides.

In another particularly preferred variant of the method, the blockers bind to the background nucleic acids and thus prevent the complete elongation of the primer oligonucleotide in the polymerase reaction.

In addition, it is preferred according to the invention that the chemically treated DNA sample is amplified in the second step with the use of a primer oligonucleotide and blocker, which hybridizes to a 5'-CG-3' dinucleotide or a 5'-TG-3' dinucleotide or a 5'-CA-3' dinucleotide, or any combinations thereof and at least one reporter oligonucleotide, which hybridizes to a 5'-CG-3' dinucleotide or a 5'-TG-3' dinucleotide or a 5'-CA-3' dinucleotide, or any combination thereof, as well as a polymerase; whereby the blocker preferably binds to the background nucleic acid and adversely affects its amplification, and whereby the reporter oligonucleotide preferably binds to the target nucleic acid (or copies thereof) and indicates its amplification. It is thus advantageous that another oligomer labeled with a fluorescent dye is used in addition to the reporter oligonucleode so that this other oligomer hybridizes directly adjacent to the

reporter oligonucleotide and this hybridization can be detected by means of fluorescence resonance energy transfer. It is further advantageous that a Molecular Beacon assay is conducted. It is also preferable that a LightCycler assay is conducted.

It is also a particularly preferred embodiment of the invention that all steps of the amplification procedure take place in one tube, or well, or other suitable vial, and that all necessary components may be added at once. This is to eliminate the chance for contamination during the amplification procedure.

In one preferred embodiment wherein no heat denaturation is performed the vial may be kept closed after having added all components necessary at once. If heat denaturation is performed (see table, steps 3, 5), the vial must be opened one or two times, to add reverse transcriptase a second time as it is not heat resistant, plus all other components required.

In embodiments wherein nucleic acids are amplified, it may be desirable to separate the amplification product from the template and the excess primer for the purpose of determining whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods (Sambrook et al., In: Molecular Cloning: A Laboratory Manual 2 rev.ed., Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989).

Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column chromatography.

Amplification products must be visualized in order to confirm

amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled, nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art. Briefly, amplification products are separated by gel electrophoresis. The gel is then contacted with a membrane, such as nitrocellulose, permitting transfer of the nucleic acid and non-covalent binding. Subsequently, the membrane is incubated with a chromophore-conjugated probe that is capable of hybridizing with a target amplification product. Detection is by exposure of the membrane to x-ray film or ion-emitting detection devices.

A particulary preferred method for detection of amplified products is use of fluorescently labeled oligonucleotides, which bind to sequences representing the methylation state to be investigated. Examples for such oligonucleotides are Molecular Beacons or LightCycler probes, which both carry dyes, which will only emit light of a defined wavelength, when they

are bound to their target. This can be measured continuously during amplification or after amplification.

It is preferred according to the invention that the sample DNA is obtained from serum or other body fluids of an individual.

In one embodiment of the invention the method is further characterized in that a primer 5'-tailed with the promoter sequence of a DNA-dependend RNA-polymerase is used. In a preferred embodiment said method is further characterized in that the sample amplification is performed in the presence of an enzyme that specifically hydrolyzes the phosphodiester bonds of a RNA which is hybridized to DNA. In a preferred embodiment of the present invention is the enzyme an endoribonuclease, especially Ribonuclease H (RNAse H).

In one embodiment of the present invention the amplification is conducted in the presence of at least one methylation-specific DNA-oligonucleotide blocker, whereby the blocker preferably does not bind, when the RNA-copyrepresents the methylation state to be detected, but preferably when RNA-copy represents background DNA. Especially preferred is the methylation-specific blocker an DNA-oligonucleotide blocker, which binds to a 5'-CG-3' dinucleotide or a 5'-TG-3' dinucleotide or a 5'-CA-3' dinucleotide or a combination thereof.

Additionally, the decomposition of these oligonucleotides by the polymerase must be prevented. This is done preferably either with the use of a polymerase without nuclease activity or preferably with the use of modified oligonucleotides, which have, for example, thioate bridges at the 5' terminal and thus are resistant to a decomposition.

In one embodyment of the present invention a T7-promoter in the 5'-region of one of the primers used will serve as the transcription initiation site of T7-RNA-polymerase as soon as

this region consist of double stranded DNA. Thereby RNA-copies are generated. DNA-oligonucleotide blocker will bind to RNA-copies depending on the methylation state their sequence is representing. If the blocker binds, RNase H will specifically degrade RNA within the heteroduplex region. If blocker does not bind RNA is available for further reactions.

Particularly preferred is the method in which a binding site of the methylation-specific DNA-oligonucleotide blocker overlaps with the binding sites of the primers on the background nucleic acid, whereby the methylation-specific DNA-oligonucleotide blocker hinders the binding of at least one primer to the background nucleic acid. Of these, preferred is the method wherein at least two additional methylation-specific DNA-oligonucleotide blocker are utilized, whereby their binding sites each overlap in turn with the binding site of one primer on the background nucleic acid, and the methylation-specific DNA-oligonucleotide blocker hinder the binding of both primer oligonucleotides to the background nucleic acid.

In one another embodiment of the present invention, the method of the invention is further characterized in that the sample DNA is obtained from body fluids of an individual. 'Body fluid' herein refers to a mixture of macromolecules obtained from an organism. This includes, but is not limited to, blood, blood plasma, blood serum, urine, sputum, ejaculate, semen, tears, sweat, saliva, lymph fluid, bronchial lavage, pleural effusion, peritoneal fluid, meningal fluid, amniotic fluid, glandular fluid, fine needle aspirates, nipple aspirate fluid, spinal fluid, conjunctival fluid, vaginal fluid, duodenal juice, pancreatic juice, bile and cerebrospinal fluid. This also includes experimentally separated fractions of all of the preceding. 'Body fluid' also includes solutions or mixtures containing homogenised solid material, such as faeces.

It is also preferred that the sample DNA is obtained from tissue sources, such as for example provided as clinical samples, for example tissue embedded in paraffin, histological slides or fresh frozen tissue. These tissues may be for example, tissue from eyes, intestine, kidneys, brain, heart, prostate, lungs, breast or liver, or all possible combinations thereof.

Another subject of the present invention is also the use of a method according to the invention for the diagnosis and/or prognosis of adverse events for patients or individuals, whereby these adverse events belong to at least one of the following categories: undesired drug interactions; cancer disorders; CNS malfunctions, damage or disease; symptoms of aggression or behavioral disturbances; clinical, psychological and social consequences of brain damage; psychotic disturbances and personality disorders; dementia and/or associated syndromes; cardiovascular disease, malfunction and damage; malfunction, damage or disease of the gastrointestinal tract; malfunction, damage or disease of the respiratory system; lesion, inflammation, infection, immunity and/or convalescence; malfunction, damage or disease of the body as an abnormality in the developmental process; malfunction, damage or disease of the skin, muscles, connective tissue or bones; endocrine and metabolic malfunction, damage or disease; headaches or sexual malfunction.

The invention also relates to the diagnosis of diseases associated with cellular immunodeficiency, e.g. a deficiency according to ICD10 Code: D.84.4. These can be septicemic diseases, inflammatory reactions and fevers, autoimmune diseases, and diseases associated with cell division disorders, such as cancer. Inflammations in the meaning of the invention are reactions of the organism, mediated by the connective tissue and blood vessels, to an external or internally

triggered inflammatory stimulus, with the purpose of eliminating or inactivating the latter and repairing the tissue lesion caused by said stimulus. A triggering effect is caused by mechanical stimuli (foreign bodies, pressure, injury) and other physical factors (ionizing radiation, UV light, heat, cold), chemical substances (alkaline solutions, acids, heavy metals, bacterial toxins, allergens, and immune complexes), and pathogens (microorganisms, worms, insects), or pathologic metabolites, derailed enzymes, malignant tumors. The process begins with a brief arteriolar constriction (as a result of adrenaline effect), with inadequate circulation and tissue alteration, followed by development of classical local inflammatory signs (cardinal symptoms, according to GALEN and CELSUS), i.e., from reddening (= rubor; vascular dilation caused by histamine), heat (= calor; as a result of local increase of metabolism), swelling (= tumor; as a result of secretion of protein-rich liquor from vessel walls changed by histamine, among other things, supported by decelerated blood circulation in the sense of a prestasis up to stasis), pain (= dolor; as a result of increased tissue tension and algogenic inflammation products, e.g. bradykinin), and functional disorders (= functio laesa). The process is accompanied by disorders in the electrolyte metabolism (transmineralization), invasion of neutrophilic granulocytes and monocytes through the vessel wall (cf., leukotaxis), with the purpose of eliminating the inflammatory stimulus and the damaged to necrotic cells (phagocytosis); furthermore, invasion of lymphocyte effector cells, giving rise to formation of specific antibodies against the inflammatory stimulus (immune reaction), and of eosinophiles (during the phase of healing or - at a very early stage - in allergic-hyperergic processes). As a result of the activation of the complement system occurring during the reaction, fragments (C3a and C5a) of this system are liberated which - like histamine and bradykinin - act as inflammation mediators, namely, in the sense of stimulating the chemotaxis of the above-mentioned blood cells; furthermore, the blood

coagulation is activated. As a consequence, damage (dystrophia and coagulation necrosis) of the associated organ parenchyma occurs. Depending on the intensity and type of the inflammation, the overall organism responds with fever, stress (cf., adaptation syndrome), leukocytosis and changes in the composition of the plasma proteins (acute-phase reaction), giving rise to an accelerated erythrocyte sedimentation. Preferred inflammations in the meaning of the invention are suppurative, exudative, fibrinous, gangrenescent, granulomatous, hemorrhagic, catarrhal, necrotizing, proliferative or productive, pseudomembranous, serous, specific and/or ulcerous inflammations.

Autoimmune diseases in the meaning of the invention are diseases entirely or partially due to the formation of autoantibodies and their damaging effect on the overall organism or organ systems, i.e., due to autoaggression. A classification into organ-specific, intermediary and/or systemic autoimmune diseases can be made. Preferred organ-specific autoimmune disease are HASHIMOTO thyroiditis, primary myxedema, thyrotoxicosis (BASEDOW disease), pernicious anemia, ADDISON disease, myasthenia gravis and/or juvenile diabetes mellitus. Preferred intermediary autoimmune diseases are GOODPASTURE syndrome, autoimmune hemolytic anemia, autoimmune leukopenia, idiopathic thrombocytopenia, pemphigus vulgaris, sympathetic ophthalmia, primary bile cirrhosis, autoimmune hepatitis, colitis ulcerosa and/or SJÖGREN syndrome. Preferred systemic autoimmune diseases are rheumatoid arthritis, rheumatic fever, systemic lupus erythematodes, dermatomyositis/polymyositis, progressive systemic sclerosis, WEGENER granulomatosis, panarteritis nodosa and/or hypersensitivity angiitis. Typical autoimmune diseases are thyrotoxicosis, thyroid-caused myxedema, HASHIMOTO thyroiditis, generalized endocrinopathy, pernicious anemia, chronic gastritis type A, diseases of single or all corpuscular elements of the blood (for example, autoimmune hemolytic

anemia, idiopathic thrombocytopenia or thrombocytopathy; idiopathic leukopenia or agranulocytosis), pemphigus vulgaris and pemphigoid, sympathetic ophthalmia, and numerous forms of uveitis, primarily biliary liver cirrhosis and chronic aggressive autoimmune hepatitis, diabetes mellitus type I, CROHN disease and colitis ulcerosa, SJÖGREN syndrome, ADDISON disease, lupus erythematodes disseminatus and discoid form of said disease, as dermatomyositis and scleroderma, rheumatoid arthritis (= primarily chronic polyarthritis), antiglomerular basement membrane nephritis. The basis is an aggressive immune reaction due to breakdown of the immune tolerance to self-determinants and a reduction of the activity of T suppressor cells (with lymphocyte marker T8) or an excess of T helper cells (with lymphocyte marker T4) over the suppressor cells; furthermore, formation of autoantigens is possible e.g. by coupling of host proteins to haptens (e.g. drugs), by ontogenetic tissue not developing until self-tolerance has developed, by protein components demasked as a result of conformational changes of proteins in connection with e.g. infection by viruses or bacteria; and by new proteins formed in connection with neoplasias.

Septicemic diseases in the meaning of the invention are diseases due to continuous or periodic invasion of pathogenic bacteria and/or their toxins from a focus of disease and their spreading on the lymph-blood route to form a general or local infection.

Septicemia in the meaning of the invention is preferably wound septicemia (phlegmon, thrombophlebitis, lymphangitis), puerperal septicemia (in case of puerperal fever), otogenic septicemia (in case of otitis media), tonsillogenic septicemia (in case of angina, peritonsillitis), cholangitic septicemia (in case of purulent cholecystitis, cholangitis), pylephlebitic septicemia (in case of pylephlebitis) umbilical septicemia (in case of omphalitis etc.), urosepticemia, as well as dental

granuloma. Septicemia in the meaning of the invention can be acute to highly acute (foudroyant), subacute (e.g. as endocarditis lenta) or chronic, and of course, can also be neonatal septicemia.

Therefore, septicemias in the meaning of the invention are all pathogenic changes in a patient which can be associated with intermittent fever and cold chills, with spleen tumor, toxic reactions or damage of the bone marrow or blood (polynuclear leukocytosis, anemia, hemolysis, thrombocytopenia) or with pathogenic reactions in the heart and vasomotor nerve (tachycardia, centralization of the blood circulation, edemas, oliguria; possibly shock) or in the digestive tract (dry, coated tongue, diarrhea), or with septicopyemia (pyemia with formation of septic infarction and metastatic abscess).

In the meaning of the invention, preferred diseases associated with a deficiency of the cellular immune system also include:

AIDS, acne, albuminuria (proteinuria), alcohol withdrawal syndrome, allergies, alopecia (loss of hair), ALS (amyotrophic lateral sclerosis), Alzheimer's disease, retinal macula senile degeneration, anemia, anxiety syndrome, anthrax (milzbrand) aortic sclerosis, occlusive arterial disease, arteriosclerosis, arterial occlusion, arteriitis temporalis, arteriovenous fistula, asthma, respiratory insufficiency, autoimmune disease, prolapsed intervertebral disc, inflammation of the peritoneum, pancreatic cancer, Becker muscular dystrophy, benign prostate hyperplasia (BPH), bladder carcinoma, hemophilia, bronchial carcinoma, breast cancer, BSE, chlamydia infection, chronic pain, cirrhosis, commotio cerebri (brain concussion), Creutzfeld-Jacob disease, intestinal carcinoma, intestinal tuberculosis, depression, diabetes insipidus, diabetes mellitus, diabetes mellitus juvenilis, diabetic retinopathy, Duchenne muscular dystrophia, duodenal carcinoma, dystrophia musculorum progressiva, dystrophia, Ebola, eczema, erectile

dysfunction, obesity, fibrosis, cervix cancer, uterine cancer, cerebral hemorrhage, encephalitis, loss of hair, hemiplegia, hemolytic anemia, hemophilia, urinary incontinence, pet allergy (animal hair allergy), skin cancer, herpes zoster, cardiac infarction, cardiac insufficiency, cardiovalvulitis, cerebral metastases, cerebral stroke, cerebral tumor, testicle cancer, ischemia, Kahler's disease (plasmocytoma), polio (poliomyelitis); rarefaction of bone, colon carcinoma, contact eczema, palsy, liver cirrhosis, leukemia, pulmonary fibrosis, lung cancer, pulmonary edema, lymph node cancer, (Morbus Hodgkin), lymphogranulomatosis, lymphoma, lyssa, gastric carcinoma, meningitis, mucoviscidosis (cystic fibrosis), multiple sclerosis (MS), myocardial infarction, neurodermitis, neurofibromatosis, neuronal tumors, kidney cancer (kidney cell carcinoma), osteoporosis, pancreas carcinoma, pneumonia, polyarthritis, polyneuropathies, potency disorders, progressive systemic sclerosis (PSS), prostate cancer, rectum carcinoma, pleurisy, craniocerebral trauma, vaginal carcinoma, sinusitis, esophagus cancer, tremor, tuberculosis, tumor pain, burns/scalds, intoxications, viral meningitis, menopause, soft-tissue sarcoma, soft-tissue tumor, cerebral blood circulation disorders, CNS tumors.

The methods provided herein are particularly deemed useful for the diagnosis of cancer including solid tumors such as skin, breast, brain, cervical carcinomas, testicular carcinomas, etc. More particularly, cancers that may be diagnosted by the methods of the invention include, but are not limited to: Cardiac: sarcoma (angiosarcoma, fibrosarcoma, rhabdomyosarcoma, liposarcoma), myxoma, rhabdomyoma, fibroma, lipoma and teratoma; Lung: bronchogenic carcinoma (squamous cell, undifferentiated small cell, undifferentiated large cell, adenocarcinoma), alveolar (bronchiolar) carcinoma, bronchial adenoma, sarcoma, lymphoma, chondromatous hamartoma, mesotheliorna; Gastrointestinal: esophagus (squamous cell carcinoma, adenocarcinoma, leiomyosarcoma, lymphoma), stomach

(carcinoma, lymphoma, leiomyosarcoma), pancreas (ductal adenocarcinoma, insulinoma, glucagonoma, gastrinoma, carcinoid tumors, vipoma), small bowel (adenocarcinoma, lymphoma, carcinoid tumors, Karposi's sarcoma, leiomyoma, hemangioma, lipoma, neurofibroma, fibroma), large bowel (adenocarcinoma, tubular adenoma, villous adenoma, hamartoma, leiomyoma); Genitourinary tract: kidney (adenocarcinoma, Wilm's tumor [nephroblastoma], lymphoma, leukemia), bladder and urethra (squamous cell carcinoma, transitional cell carcinoma, adenocarcinoma), prostate (adenocarcinoma, sarcoma), testis (seminoma, teratoma, embryonal carcinoma, teratocarcinoma, choriocarcinoma, sarcoma, interstitial cell carcinoma, fibroma, fibroadenoma, adenomatoid tumors, lipoma); Liver: hepatoma (hepatocellular carcinoma), cholangiocarcinoma, hepatoblastoma, angiosarcoma, hepatocellular adenoma, hemangioma; Bone: osteogenic sarcoma (osteosarcoma), fibrosarcoma, malignant fibrous histiocytoma, chondrosarcoma, Ewing's sarcoma, malignant lymphoma (reticulum cell sarcoma), multiple myeloma, malignant giant cell tumor chordoma, osteochronfroma (osteocartilaginous exostoses), benign chondroma, chondroblastoma, chondromyxofibroma, osteoid osteoma and giant cell tumors; Nervous system: skull (osteoma, hemangioma, granuloma, xanthoma, osteitis deformans), meninges (meningioma, meningiosarcoma, gliomatosis), brain (astrocytoma, medulloblastoma, glioma, ependymoma, germinoma [pinealoma], glioblastoma multiform, oligodendroglioma, schwannoma, retinoblastoma, congenital tumors), spinal cord neurofibroma, meningioma, glioma, sarcoma); Gynecological: uterus (endometrial carcinoma), cervix (cervical carcinoma, pre-tumor cervical dysplasia), ovaries (ovarian carcinoma [serous cystadenocarcinorna, mucinous cystadenocarcinoma, unclassified carcinoma], granulosa-thecal cell tumors, Sertoli-Leydig cell tumors, dysgerminoma, malignant teratoma), vulva (squamous cell carcinoma, intraepithelial carcinoma, adenocarcinoma, fibrosarcoma, melanoma), vagina (clear cell carcinoma, squamous cell carcinoma, botryoid sarcoma (embryonal rhabdomyosarcoma],

fallopian tubes (carcinoma); Hematologic: blood (myeloid leukemia [acute and chronic], acute lymphoblastic leukemia, chronic lymphocytic leukemia, myeloproliferative diseases, multiple myeloma, myelodysplastic syndrome), Hodgkin's disease, non-Hodgkin's lymphoma [malignant lymphoma]; Skin: malignant melanoma, basal cell carcinoma, squamous cell carcinoma, Karposi's sarcoma, moles dysplastic nevi, lipoma, angioma, dermatofibroma, keloids, psoriasis; and Adrenal glands: neuroblastoma. Thus, the term "cancerous cell" as provided herein, includes a cell afflicted by any one of the above identified conditions.

In a preferred embodiment the cancerous disease or tumor being diagnosted is selected from the group of cancerous diseases or tumor diseases of the ear-nose-throat region, of the lungs, mediastinum, gastrointestinal tract, urogenital system, gynecological system, breast, endocrine system, skin, bone and soft-tissue sarcomas, mesotheliomas, melanomas, neoplasms of the central nervous system, cancerous diseases or tumor diseases during infancy, lymphomas, leukemias, paraneoplastic syndromes, metastases with unknown primary tumor (CUP syndrome), peritoneal carcinomatoses, immunosuppression-related malignancies and/or tumor metastases. More specifically, the tumors may comprise the following types of cancer: adenocarcinoma of breast, prostate and colon; all forms of lung cancer starting in the bronchial tube; bone marrow cancer, melanoma, hepatoma, neuroblastoma; papilloma; apudoma, choristoma, branchioma; malignant carcinoid syndrome; carcinoid heart disease, carcinoma (for example, Walker carcinoma, basal cell carcinoma, squamobasal carcinoma, Brown-Pearce carcinoma, ductal carcinoma, Ehrlich tumor, in situ carcinoma, cancer-2 carcinoma, Merkel cell carcinoma, mucous cancer, non-parvicellular bronchial carcinoma, oat-cell carcinoma, papillary carcinoma, scirrhus carcinoma, bronchio-alveolar carcinoma, bronchial carcinoma, squamous cell carcinoma and transitional cell carcinoma); histiocytic functional disorder;

leukemia (e.g. in connection with B cell leukemia, mixed-cell leukemia, null cell leukemia, T cell leukemia, chronic T cell leukemia, HTLV-II-associated leukemia, acute lymphocytic leukemia, chronic lymphocytic leukemia, mast cell leukemia, and myeloid leukemia); malignant histiocytosis, Hodgkin disease, non-Hodgkin lymphoma, solitary plasma cell tumor; reticuloendotheliosis, chondroblastoma; chondroma, chondrosarcoma; fibroma; fibrosarcoma; giant cell tumors; histiocytoma; lipoma; liposarcoma; leukosarcoma; mesothelioma; myxoma; myxosarcoma; osteoma; osteosarcoma; Ewing sarcoma; synovioma; adenofibroma; adenolymphoma; carcinosarcoma, chordoma, craniopharyngioma, dysgerminoma, hamartoma; mesenchymoma; mesonephroma, myosarcoma, ameloblastoma, cementoma; odontoma; teratoma; thymoma, chorioblastoma; adenocarcinoma, adenoma; cholangioma; cholesteatoma; cylindroma; cystadenocarcinoma, cystadenoma; granulosa cell tumor; gynadroblastoma; hidradenoma; islet-cell tumor; Leydig cell tumor; papilloma; Sertoli cell tumor, theca cell tumor, leiomyoma; leiomyosarcoma; myoblastoma; myoma; myosarcoma; rhabdomyoma; rhabdomyosarcoma; ependymoma; ganglioneuroma, glioma; medulloblastoma, meningioma; neurilemmoma; neuroblastoma; neuroepithelioma, neurofibroma, neuroma, paraganglioma, non-chromaffin paraganglioma, angiokeratoma, angiolymphoid hyperplasia with eosinophilia; sclerotizing angioma; angiomatosis; glomangioma; hemangioendothelioma; hemangioma; hemangiopericytoma, hemangiosarcoma; lymphangioma, lymphangiomyoma, lymphangiosarcoma; pinealoma; cystosarcoma phylloides; hemangiosarcoma; lymphangiosarcoma; myxosarcoma, ovarial carcinoma; sarcoma (for example, Ewing sarcoma, experimentally, Kaposi sarcoma and mast cell sarcoma); neoplasms (for example, bone neoplasms, breast neoplasms, neoplasms of the digestive system, colorectal neoplasms, liver neoplasms, pancreas neoplasms, hypophysis neoplasms, testicle neoplasms, orbital neoplasms, neoplasms of the head and neck, of the central nervous system, neoplasms of the hearing organ, pelvis, respiratory tract and urogenital tract);

neurofibromatosis and cervical squamous cell dysplasia. In another preferred embodiment the cancerous disease or tumor being diagnosted is selected from the group of tumors of the ear-nose-throat region, comprising tumors of the inner nose, nasal sinus, nasopharynx, lips, oral cavity, oropharynx, larynx, hypopharynx, ear, salivary glands, and paragangliomas, tumors of the lungs comprising non-parvicellular bronchial carcinomas, parvicellular bronchial carcinomas, tumors of the mediastinum, tumors of the gastrointestinal tract, comprising tumors of the esophagus, stomach, pancreas, liver, gallbladder and biliary tract, small intestine, colon and rectal carcinomas and anal carcinomas, urogenital tumors comprising tumors of the kidneys, ureter, bladder, prostate gland, urethra, penis and testicles, gynecological tumors comprising tumors of the cervix, vagina, vulva, uterine cancer, malignant trophoblast disease, ovarial carcinoma, tumors of the uterine tube (Tuba Faloppii), tumors of the abdominal cavity, mammary carcinomas, tumors of the endocrine organs, comprising tumors of the thyroid, parathyroid, adrenal cortex, endocrine pancreas tumors, carcinoid tumors and carcinoid syndrome, multiple endocrine neoplasias, bone and soft-tissue sarcomas, mesotheliomas, skin tumors, melanomas comprising cutaneous and intraocular melanomas, tumors of the central nervous system, tumors during infancy, comprising retinoblastoma, Wilms tumor, neurofibromatosis, neuroblastoma, Ewing sarcoma tumor family, rhabdomyosarcoma, lymphomas comprising non-Hodgkin lymphomas, cutaneous T cell lymphomas, primary lymphomas of the central nervous system, morbus Hodgkin, leukemias comprising acute leukemias, chronic myeloid and lymphatic leukemias, plasma cell neoplasms, myelodysplasia syndromes, paraneoplastic syndromes, metastases with unknown primary tumor (CUP syndrome), peritoneal carcinomatosis, immunosuppression-related malignancy comprising AIDS-related malignancy such as Kaposi sarcoma, AIDS-associated lymphomas, AIDS-associated lymphomas of the central nervous system, AIDS-associated morbus Hodgkin and AIDS-associated anogenital tumors, transplantation-related

malignancy, metastasized tumors comprising brain metastases, lung metastases, liver metastases, bone metastases, pleural and pericardial metastases, and malignant ascites.

The use of a method according to the invention is thus advantageous for distinguishing cell types or tissues or for investigating cell differentiation.

The subject of the present invention is also a kit, comprised of a reagent containing a bisulfite, primers, one 5'-tailed with a promoter seqeunce for RNA-polymaerase a methylation-specfic blocker, RNA-polymerase, RNase H, (reverse transcriptase) as well as optionally, instructions for conducting an assay according to the invention. The kit may also include information (instruction leaflet, internet address) explaining how to combine the components of the kit. Said information can also be related to a diagnostic scheme.

The present invention thus describes a method for the detection of the methylation state of genomic DNA samples. In contrast to previously known methods, the degree of methylation of a set of CpG positions is determined in a selected subgroup of DNA fragments, e.g. in serum, so that an analysis is also possible in the presence of an excess of diagnostically irrelevant background DNA.

The Table 1 illustrates a preferred method of the present invention, which are not intended to limit the scope of the invention thereto.

As will be apparent to those skilled in the art in which the invention is addressed, the present invention may be embodied in forms other than those specifically disclosed above without departing from the spirit or essential characteristics of the invention. The particular embodiments of the present invention, described above, are therefore to be considered in all respects

as illustrative and not restrictive. The scope of the present invention is as set forth in the appended claims rather than being limited to the examples contained in the foregoing description.

CLAIMS

1. A method for the detection of cytosine methylation in a DNA sample comprising the steps:

- a genomic DNA sample, which comprises the DNA to be investigated as well as background DNA is chemically treated in such a way that all of the unmethylated cytosine bases are converted to uracil, while the 5-methylcytosine bases remain unchanged;

- the chemically treated DNA sample is amplified by using a nucleic acid amplification method including a transcription step, whereby the amplification is conducted in the presence of at least one methylation-specific blocker, which binds to the background nucleic acid but substantially does not bind to the target nucleic acid, and

- the amplified products are analyzed and the methylation status in the DNA to be investigated is concluded from the presence or absence of an amplified product and/or from the analysis of additional CpG positions.

2. Method according to claim 1, whereby the said amplification method is selected from the group comprising a self-sustaining sequence replication (3SR), a nucleic acid sequence based amplification (NASBA), a transcription-mediated amplification (TMA) or transcription from an artificially introduced promoter sequence.

3. Method according to one of the preceding claims, further characterized in that a primer 5'-tailed with the promoter sequence of a DNA-dependent RNA-polymerase is used.

4. Method according to one of the preceding claims, further characterized in that the sample amplification is performed in the presence of an enzyme that specifically hydrolyzes the phosphodiester bonds of a RNA which is hybridized to DNA.

5. Method according to one of the preceding claims, further characterized in that the enzyme is a endoribonuclease, especially Ribonuclease H (RNAse H).

6. The method according to one of the preceding claims, further characterized in that the methylation-specific blocker is a DNA-oligonucleotide blocker, which binds to at least one or several dinucleotides selected from the group comprising a 5'-CG-3' dinucleotide, a 5'-TG-3' dinucleotide and a 5'-CA-3' dinucleotide.

7. The method according to one of the preceding claims, further characterized in that the blockers are present modified at the 5' end and thus cannot be significantly broken down by a polymerase with 5'-3' exonuclease activity.

8. The method according to one of the preceding claims, further characterized in that the blockers that are used in addition to the primers do not make available a 3'-OH function.

9. The method according to one of the preceding claims, further characterized in that a binding site of the methylation-specific DNA-oligonucleotide blocker overlaps with the binding sites of a primer on the background nucleic acid, whereby the methylation-specific DNA-oligonucleotide blocker hinders the binding of at least one primer to the background DNA.

10. The method according to one of the preceding claims, further characterized that at least two additional methylation-specific DNA-oligonucleotide blocker are utilized, whereby their binding sites each overlap in turn with the binding site of one primer on the background nucleic acid, and the methylation-specific DNA-oligonucleotide blocker hinder the binding of both primer oligonucleotides to the background nucleic acid.

11. The method according to one of the preceding claims, further characterized in that said DNA sample is obtained from a body fluid of an individual.

12. The method according to one of the preceding claims, further characterized in that said body fluid is one or several out of the group comprising blood, blood plasma, blood serum, urine, sputum, ejaculate, semen, tears, sweat, saliva, lymph fluid, bronchial lavage, pleural effusion, peritoneal fluid, meningal fluid, amniotic fluid, glandular fluid, fine needle aspirates, nipple aspirate fluid, spinal fluid, conjunctival fluid, vaginal fluid, duodenal juice, pancreatic juice, bile, cerebrospinal fluid and faeces.

13. The method according to one of the preceding claims, further characterised in that said DNA sample is obtained from

a tissue obtained from a clinical samples, a tissue embedded in paraffin, histological slides or from fresh frozen tissue.

14. The method according to one of the preceding claims, further characterised in that said tissue is one out of the group comprising tissues from eyes, intestine, kidneys, brain, heart, prostate, lungs, breast and liver, or all possible combinations thereof.

15. The method according to one of the preceding claims, further characterized in that the chemical treatment is conducted with a bisulfite (= disulfite, hydrogen sulfite).

16. The method according to one of the preceding claims, further characterized in that the chemical treatment is conducted after embedding the DNA in agarose.

17. The method according to one of the preceding claims, further characterized in that a reagent denaturing the DNA duplex is present in the chemical treatment.

18. Kit comprising a reagent containing a bisulfite, primers, one of them 5'-tailed with a promoter sequence for RNA-polymerase, a methylation-specfic blocker, RNase H, DNA dependent RNA-polymerase, as well as optionally, an information about the using of parts of the kit.

ABSTRACT

The present invention relates generally to a method of determining the DNA methylation status of CpG sites in a given locus. In particular, this invention relates to the use of DNA amplification techniques for the detection of cytosin methylation patterns, especially to the use of DNA-NASBA (Nucleic Acid Sequence Based Amplification) for sensitive detection of DNA-methylation.

# PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

# PCT

NOTIFICATION CONCERNING
SUBMISSION OR TRANSMITTAL
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

SCHUBERT, Klemens
Neue Promenade 5
10178 Berlin
ALLEMAGNE

| | |
|---|---|
| Date of mailing *(day/month/year)*<br>10 June 2005 (10.06.2005) | |
| Applicant's or agent's file reference<br>P1437PC00 | **IMPORTANT NOTIFICATION** |
| International application No.<br>PCT/EP05/003366 | International filing date *(day/month/year)*<br>24 March 2005 (24.03.2005) |
| International publication date *(day/month/year)* | Priority date *(day/month/year)*<br>24 March 2004 (24.03.2004) |
| Applicant<br>EPIGENOMICS AG et al | |

1. By means of this Form, which replaces any previously issued notification concerning submission or transmittal of priority documents, the applicant is hereby notified of the date of receipt by the International Bureau of the priority document(s) relating to all earlier application(s) whose priority is claimed. Unless otherwise indicated by the letters "NR", in the right-hand column or by an asterisk appearing next to a date of receipt, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

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| Priority date | Priority application No. | Country or regional Office or PCT receiving Office | Date of receipt of priority document |
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| 24 March 2004 (24.03.2004) | 04090117.5 | EP | 25 May 2005 (25.05.2005) |
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| Date of mailing *(day/month/year)*<br>31 May 2005 (31.05.2005) | |
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